Physiological and Cytogenetic Characterization of Immortalized Human Endometriotic Cells Containing Episomal Simian Virus 40 DNA

Ali Akoum,* Josée Lavoie,[†] Régen Drouin,[†]
Christine Jolicoeur,* André Lemay,*
Rodolphe Maheux,* and Edouard W. Khandjian[†]

From the Laboratoire d'Endocrinologie de la Reproduction* and
Unité de Recherche en Génétique Humaine et Moléculaire,[†]
Centre de Recherche, Pavillon Saint-François d'Assise, Centre
Hospitalier Universitaire de Québec, Université Laval,
Québec, Canada

The study of misplaced endometrial cells, which abnormally implant and grow outside the uterine cavity, is of considerable interest for the understanding of the pathophysiology of endometriosis. However, endometriotic cells, particularly epithelial cells, required for primary cell culture are not easily available. We report here the characterization of an endometriotic cell line immortalized after infection of primary endometriotic cell cultures with simian virus 40. Transformed cells express T-antigen, and blot hybridization analysis showed that the viral genome is present as an episome. Cytogenetic analysis revealed a polyploid karyotype with numerical and structural rearrangements involving mainly the same chromosomes (6, 10, 11, 15, and 17). The cell line has been maintained in culture for over 80 passages and was still proliferating without any noticeable change in the biological properties investigated. Transformed endometriotic cells expressed both progesterone and estradiol receptors and were stimulated by these ovarian hormones to secrete monocyte chemotactic protein-1, a factor that may play an important role in the recruitment and activation of peritoneal macrophages. In addition, this response was enhanced in interleukin-1-treated cells. Taken together, these findings support the view that this cell line may be an interesting tool for the study of the pathophysiology of endometriosis. (*Am J Pathol* 1999, 154:1245-1257)

Endometriosis is a gynecological disorder defined as an ectopic development of endometrial-like tissue, which is commonly found on the organs of the pelvis. A series of biological changes, including an increased number of activated macrophages^{1,2} and elevated levels of cytokines,³⁻⁷ prostaglandins,⁸ enzymes,² and complement components⁹ have frequently been observed in the peri-

toneal cavity of patients having endometriosis. These complex responses might be involved in endometriosis-associated pain and infertility.^{2,3,5} Ectopic endometrial tissue is biologically active, particularly in the milder stages of the disease,⁸ and may have a significant role in the alteration of the peritoneal environment. Endometriotic implants have the capacity to produce prostaglandins⁸ and vascular endothelium growth factor (VEGF).¹⁰ They also synthesize and secrete complement component 3, which possesses a chemotactic activity for macrophages.⁹ Recently, we have shown that endometriotic cells secrete monocyte chemotactic protein (MCP)-1, a cytokine that could be involved in monocyte activation and recruitment into the peritoneal cavity of patients.¹¹ We have also shown that endometriotic cells secrete high levels of interleukin (IL)-6,¹² and, according to other studies, these cells exhibit an altered responsiveness to IL-6 as compared with uterine endometrial cells.¹³

The biological properties of ectopic endometrial cells have been poorly investigated, mainly due to the rare availability of endometriotic tissue required for cell culture and the limited number of cells, particularly those of epithelial type, that could be isolated from the tissue.

In this report, we describe the development of a human endometriotic cell line that was immortalized by the simian virus (SV)40. The cell line has a polyploid karyotype, is of epithelial-like nature, and was maintained in culture for over 80 passages without any sign of senescence. All immortalized cells express T-antigen as seen by indirect immunofluorescence staining and contain SV40 DNA in an episomal form. They also express receptors for ovarian steroids, retain differentiated functions, and respond to these latter and to the pro-inflammatory cytokine IL-1 β by secreting MCP-1 in a manner comparable to that of primary endometriotic epithelial cells.

Supported by grants MT-12541 and MT-14638 from the Medical Research Council of Canada to A. Akoum. J. Lavoie holds a studentship from a shared contribution from the Division of Pathology of the Department of Medical Biology and the Faculty of Medicine, Laval University, and R. Drouin is a research scholar of the Cancer Research Society Inc./Medical Research Council of Canada program. A. Akoum is a Chercheur-Boursier Senior of the Fonds de la Recherche en Santé du Québec.

Accepted for publication December 22, 1998.

Address reprint requests to Dr. Ali Akoum, Laboratoire d'Endocrinologie de la Reproduction, Centre de Recherche, Pav. Saint-François d'Assise, 10 rue de l'Espinay, Québec, Québec, Canada, G1L 3L5. E-mail: ali.akoum@crsfa.ulaval.ca.

Table 1. Patient Characteristics at the Time of Laparoscopy

Patient	Age (years)	Cycle phase	Infertility (years)	Pain	Stage of endometriosis	Location of endometriotic tissue
1	30	Follicular	5	Dysmenorrhea	II	Peritoneum (red vesicle)
2	38	Luteal	No	Dyspareunia	II	Peritoneum (red vesicle)
3	38	Luteal	No	Dyspareunia	III	Right ovary (endometrioma)

Materials and Methods

Source and Handling of Tissue

Tissue specimens used in this study were obtained from three women with endometriosis who had given informed consent before laparoscopy. Two women had revised American Fertility Society (rAFS) stage II and one had stage III endometriosis. Age, cycle phase (determined according to the regularity of the cycle and the date of the previous menses), infertility, pain, stage of endometriosis, and location of endometriotic tissue were the main clinical characteristics listed in Table 1. Ovarian endometrioma cyst lining and endometriotic foci were placed at 4°C in sterile Hanks' balanced salt solution (HBSS) containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin and were transported to the laboratory.

Tissue Dissociation and Cell Culture

Endometriotic tissue was minced into small pieces and treated with collagenase to dissociate epithelial glands from fibroblast-like cells, which were further separated by differential sedimentation and adhesion as previously described.¹¹ The purity of epithelial cells was assessed morphologically by light microscopy and immunocytochemically with specific monoclonal antibodies to cytokeratins as described below. Cells in primary cultures were propagated and maintained in Dulbecco's modified Eagle's medium (DMEM)-F12 medium containing antibiotics and 10% fetal bovine serum (FBS).

Establishment of SV40-Immortalized Cell Lines

Primary cultures of endometriotic epithelial cells isolated from patient 1 (Table 1) and grown in a 16-mm-diameter well (24-well plate; Costar Corp., Bedford, MA) were infected with SV40 wild-type strain (SV40 wt 776) at an input multiplicity of 25 to 50 plaque-forming units (PFU) per cell as used for infection of nonpermissive mouse cells¹⁴ and maintained without FBS for 48 hours. Thereafter, cultures were passaged at a split ratio of 1:5, subcultures were maintained for 6 weeks in DMEM-F12 containing 10% FBS, and the medium was changed at 3-day intervals. Surviving cells from individual foci were cloned by limiting dilution in microtiter wells. Each clonal stock was then passaged through at least two single-cell isolation procedures, and cells were grown into continuous cell lines after being passaged with a 1:5 split ratio. Finally, six cell lines were retained, and only one, called

clone 3 (Clo03), has been selected for the present investigation.

Southern Blot Analysis

High-molecular-weight DNA was isolated from SV40-immortalized endometrial cells after purification of nuclei in the presence of 0.5% Nonidet P-40 followed by digestion with 20 mg/ml proteinase K in the presence of 1% SDS according to the method of Gross-Bellard et al.¹⁵ To isolate episomal SV40 DNA, purified nuclei were resuspended in an isotonic buffer composed of 20 mmol/L Tris/HCl, pH 7.4, 136 mmol/L NaCl, 5 mmol/L KCl, and 1 mmol/L Na₂HPO₄, and viral DNA was extracted after vigorous homogenization with a Dounce homogenizer using 30 to 40 strokes followed by elimination of nuclei after centrifugation at low speed.¹⁶ The supernatant containing the viral DNA was then digested with proteinase K, phenol extracted, and precipitated with 2 vol of ethanol at -20°C for 18 hours. Approximately 10 µg of DNA was digested overnight at 37°C with the appropriate restriction enzyme following the manufacturer's recommendations (New England BioLabs, Beverly, MA). Restriction products were resolved by electrophoresis through 0.8% agarose gels in TAE (40 mmol/L Tris/HCl, pH 7.9, 20 mmol/L sodium acetate, 10 mmol/L EDTA) buffer, and the DNA fragments were transferred onto Qiabran positively charged Nylon Plus membranes (Qiagen, Mississauga, Ontario, Canada) by vacuum blotting and fixed to the membrane by ultraviolet irradiation.¹⁷ Hybridization was performed using SV40 DNA extracted from virions and purified by CsCl/ethidium bromide gradient and labeled to high specific activity with [α^{32} P]dCTP using the ¹⁷QuickPrime kit (Pharmacia Biotech, Piscataway, NJ) in the conditions described previously,¹⁷ and the membranes were exposed to Kodak X-OMAT AR films (Eastman Kodak Co., Rochester, NY) in the presence of an intensifying screen at -70°C.

Analysis of the SV40 Replication Origin (Ori) Sequence

Ori was amplified by the polymerase chain reaction (PCR) using DNA extracted from the immortalized endometriotic cells and from purified SV40 DNA. Either 500 ng of cell DNA or 200 ng of SV40 DNA was used as template for amplification in a final volume of 100 µl containing 2 mmol/L MgCl₂, 5 U of *Taq* polymerase, 0.2 mmol/L dNTPs, and 100 nmol/L of each primer (5'AAATACCTCAGTTGCATCCCAGAAGCCTCC3' and 5'AATGTGTGT-

CAGTTAGGGTGTGGAAAGTCC3'; amplicon size, 538 bp). DNA was first denatured for 3 minutes at 98°C, and amplification carried out for 35 cycles of 2 minutes annealing at 58°C, 3 minutes extension at 74°C, and 1 minute denaturation at 98°C followed by a final extension of 10 minutes at 74°C. PCR products were analyzed by agarose gel electrophoresis and detected after ethidium bromide staining. The generated fragments were purified using the QIAquick gel extraction kit (Qiagen) and sequenced using an automated sequencer (373 DNA Sequencer Stretch with 8XL upgrade, Applied Biosystems, Perkin Elmer, Foster City, CA) and BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Perkin Elmer).

Immunofluorescence Analyses

Cells grown on coverslips were fixed in a mixture of acetone, methanol and formaldehyde (19:19:2 v/v) for 10 minutes at -20°C and exposed to the first antibody for 1 hour at room temperature. Antigen-antibody complexes were revealed by incubation for 1 hour at room temperature with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulins (1/50 dilution in PBS). SV40 large T-antigen expression in immortalized endometriotic cells was detected using mouse monoclonal antibodies PAb 414 or 419.¹⁸ The phenotype of immortalized cells was examined using mouse monoclonal antibodies specific to cytokeratins (AE1:AE3 Mix, ICN Biomedicals, St-Laurent, Quebec, Canada), vimentin (Sigma), and a 200-kd glycoprotein expressed by endometrial/endometriotic epithelial cells¹⁹ (BMA 180 mAb, also known as BW 495/36, a gift from Dr. Peter Gronski, Centeon Pharma, Marburg, Germany). In each series of analysis, specimens incubated without the primary antibody were included as controls. Samples were mounted in Mowiol-0.1% *p*-phenylenediamine and observed with a Leica microscope (Leica Mikroskopie und Systeme, Wetzlar, Germany) equipped for epifluorescence illumination.

Western Blot Analysis

Total proteins were extracted in SDS sample buffer (68 mmol/L Tris/HCl, pH 9.0, 2% β -mercaptoethanol, 0.01% bromophenol blue, and 15% glycerol) and heat denatured in a boiling bath for 3 minutes. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) in 7.5% to 15% acrylamide linear gradient slab gels and then electrotitrated onto 0.45- μ m nitrocellulose membranes. T-antigen was detected using monoclonal antibodies PAb 414 or 419 followed by horseradish-peroxidase-conjugated sheep anti-mouse immunoglobulins (Amersham Canada, Ockville, Ontario, Canada), and the immunocomplexes were revealed by chemiluminescence (RPN 2109, Amersham) as previously described.²⁰ Membranes were exposed to Kodak XAR films for 1 to 3 minutes.

Chromosome Analysis

Cells at the exponential phase of growth were arrested by adding 0.03 μ g/ml colchicine for 2 to 4 hours and treated with 10 μ g/ml ethidium bromide to prevent chromosome condensation and to increase banding resolution.²¹ Cells were then harvested, treated with a hypotonic solution (75 mmol/L KCl) at pH 8.0 for 20 minutes at 37°C, and fixed three times in cold Carnoy's fixative (3 vol of methanol/1 vol of acetic acid) for 15 minutes.²² For cells at culture passage 13, chromosomes of 113 mitoses were counted after GTG banding (G-bands by Trypsin using Giemsa),²² and 30 metaphases were photographed and analyzed. For passage 78, chromosomes of 55 metaphases were counted.

Molecular Cytogenetics

The following probes were used: digoxigenin-labeled coasome 6, 11, and 19 total chromosome probes (Oncor, Gaithersburg, MD). The slides containing chromosome spreads were soaked in 2X SSC (0.15 mol/L sodium chloride, 0.015 mol/L sodium citrate) pH 7, at 37°C for 1 hour and dehydrated in a 70%/80%/100% ethanol series. Just before hybridization the chromosome preparation was denatured in 70% (v/v) formamide/2X SSC, pH 7, for 90 seconds at 70°C and then dehydrated in ethanol at 4°C. The hybridization mixture (total volume of 10 μ l containing the specific probe) was then denatured at 70°C for 10 minutes and incubated for 2 hours at 37°C before being incubated overnight in a humidified chamber at 37°C with the chromosome preparation. Slides were then washed at 43°C with 50% formamide/2X SSC for 10 minutes then with 2X SSC for 10 minutes and finally twice in PBD (Oncor) for 5 minutes at room temperature. Sites of hybridization were visualized by indirect immunofluorescence. First, 100 μ l of a 1:50 dilution of mouse monoclonal anti-digoxigenin antibody (Boehringer Mannheim, Laval, Quebec, Canada) for coasome detection was added, followed by incubation in a humidified chamber at 37°C for 45 minutes. Slides were washed twice for 5 minutes in PBD at room temperature. A second incubation was carried out for 30 minutes at 37°C with 100 μ l of a 1:50 dilution of digoxigenin-conjugated sheep anti-mouse IgG (Boehringer Mannheim) and biotin-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA), followed by two washes. Slides were then incubated for 30 minutes at 37°C with 100 μ l of a 1:100 dilution of sheep anti-digoxigenin-rhodamine Fab fragments (Boehringer Mannheim) and streptavidin-FITC (Gibco BRL, Burlington, Ontario, Canada), followed by two washes. The slides were then stained with 125 ng/ml 4,6-diamino-2-phenylindole (DAPI) II (Vysis, Downers Grove, IL) for 5 minutes at room temperature. All pictures were taken with an Olympus BX60 microscope equipped with an image analysis system using a black and white digital camera (IMAC-CCD 930) coupled with the *in situ* imaging system (ISIS 2) software version 2.5 (Metasystems, Belmont, MA). Separate filter sets were used to view hybridization signals of chromosomes, and the images were merged using the image analysis system.

Estimation of Doubling Time

Cells were seeded at an initial concentration of 15×10^3 cells/cm² in six-well multiplates and cultured in DMEM-F12 medium supplemented with 10% FBS, 10 μ g/ml insulin, and 5 μ g/ml transferrin. The culture medium was refreshed 2 hours after plating (to discard non-adhering cells) and every 48 hours. Cells were dissociated with trypsin/EDTA and counted in triplicate, first after the 2-hour adhesion ($t = 0$) and then at different time intervals for a total of 168 hours.

Culture Stimulation and MCP-1 Secretion

For stimulation with IL-1 β cells were seeded at 50,000 cells/well in 24-well tissue culture plates (Costar) and allowed to grow to confluence in RPMI medium containing 10 μ g/ml insulin and 5 μ g/ml transferrin and supplemented with 10% dextran-coated charcoal-treated FBS (FBS-DC). MCP-1 secretion was evaluated by immunoprecipitation and SDS-PAGE after metabolic labeling with [³⁵S]cysteine (Amersham) and by enzyme-linked immunosorbent assay (ELISA) according to our previously reported procedures.^{11,17}

For treatment with progesterone (P; 4-pregnen-3,20-dione) and estradiol (E₂; (1,3,5,(10)-estratrien-3,17 β -diol 3-benzoate) (Sigma), cells were seeded at 20×10^4 cells/well in 24-well plates and allowed to adhere for 2 hours at 37°C/5% CO₂. The culture medium was then removed and replaced with a fresh medium containing different concentrations of hormones. Cells were maintained in culture for 7 to 8 days (until confluence), and the media were changed every 2 days. At confluence, cells were washed with serum-free RPMI, and incubation with hormones was pursued in this medium for an additional 42 hours. Finally, cells were exposed or not to IL-1 β , which was added to the culture medium to reach a final concentration of 1 ng/ml. Six hours later, the culture supernatants were collected and kept in small aliquots at -80°C until use for MCP-1 assay by ELISA.

For treatment with dexamethasone (Sigma), cells grown to confluence were incubated with serum-free RPMI for 18 hours and then with dexamethasone for 24 hours in the serum-free RPMI before exposure to IL-1 β for an additional 6 hours. Culture supernatants were then recovered, and MCP-1 levels were measured by ELISA.

Expression of P and E₂ Receptors by Immortalized Endometriotic Cells

The level of P and E₂ receptors was determined according to a ligand technique previously reported by Asselin et al, with slight modifications.²³ The assays were performed in triplicate. Briefly, cells (13×10^6) were diluted in 2 ml of buffer A (25 mmol/L Tris/HCl, 1.5 mmol/L EDTA, 10 mmol/L α -monothioglycerol, 10% glycerol, 10 mmol/L sodium molybdate, pH 7.4), sonicated on ice, and centrifuged at $105,000 \times g$ for 90 minutes, and the supernatant (cytosol) was immediately used for assay. P binding was measured using the dextran-coated charcoal (DCC)

adsorption technique. A 100- μ l volume of cytosol was mixed with 100 μ l of buffer B (10 mmol/L Tris/HCl, 1.5 mmol/L EDTA, 10 mmol/L α -monothioglycerol, pH 7.4) and 100 μ l of [17 α -methyl-³H]promegestone ([³H]R5020; specific activity, 84 Ci/mmol; New England Nuclear, Lachine, Quebec, Canada), which was used at a final concentration of 5 nmol/L in combination with 1 μ mol/L dexamethasone to mask the glucocorticoid receptors. Nonspecific binding was determined by incubating 100 μ l of cytosol with 100 μ l of [³H]R5020 and 100 μ l of unlabeled R5020 (3 μ mol/L in buffer B). The assay was ended by adding 300 μ l of DCC (1% Norit A and 0.1% dextran T-70 in buffer B), and after 10 minutes of incubation, tubes were centrifuged at $2000 \times g$ for 10 minutes, the supernatant was recovered, and radioactivity was measured by liquid scintillation spectrometry using a Beckman counter with a counting efficiency of 35%.

For E₂ receptors, [2,4,6,7-³H]17 β -estradiol ([³H]E₂; specific activity, 85 Ci/mmol; New England Nuclear) was added to the cytosol solution at a final concentration of 5 nmol/L, and unlabeled E₂ was used to determine nonspecific binding as described earlier for P receptors. Unbound steroids were separated by adding 300 μ l of hydroxylapatite (HAP; 0.25 g/ml of 50 mmol/L Tris/HCl, 10 mmol/L KH₂PO₄ buffer, pH 7.4). After 40 minutes of incubation at room temperature, tubes were centrifuged, the pellet was washed in the Tris buffer, and the radioactivity from the HAP pellet was then extracted twice with 1 ml of ethanol and counted using a Beckman counter with a counting efficiency of 35%.

The number of P and E₂ receptors was calculated with the following equation: $(SB \times V \times N \times 10^{-15}) / (CE \times 2.22 \times SA \times v \times n)$, where SB is specific binding (cpm), V is total cytosol volume (2 ml), N is Avogadro's number (6.023×10^{23}), CE is counting efficiency (0.35), SA is specific activity of [³H]P or [³H]E₂ (85 Ci/mmol), v is cytosol sample volume (0.1 ml), and n is cell number (13×10^6); 10^{-15} is used to transform femtomoles into moles, and 2.22 is a coefficient used to convert cpm into Ci.

Statistical Analyses

The results of MCP-1 secretion were expressed as mean $\pm$ SEM. A one-way analysis of variance (ANOVA) was used to determine whether there were any differences in MCP-1 levels found in the culture medium of cells after treatment with different concentrations of a cytokine or a hormone or after exposure to a cytokine for different periods of time. The Tukey's honestly significant difference test (HSD) was used *post hoc* for multiple comparisons. A probability level of less than 0.05 was considered as statistically significant.

Results

Establishment and Characterization of a SV40-Immortalized Endometriotic Cell Line

Human endometriotic tissue was isolated from the peritoneum of women with endometriosis, and primary cul-

Figure 1. T-antigen expression in SV40-immortalized endometrial cells as seen by indirect immunofluorescence staining for T-antigen (A; magnification, $\times 1353$) and by immunoblot analysis (B).

tures of endometrial epithelial cells were maintained as described in Materials and Methods. To establish stable epithelial cell lines that might maintain some differentiated characteristics of primary cultures, the SV40 large T-antigen was chosen as an immortalizing agent. Infection with SV40 was used for immortalization as our previous attempts to immortalize endometrial cells with different vectors encoding large T-antigen have failed. We infected subconfluent cultures to allow several rounds of cellular division to occur after infection to increase the probability of viral integration into the host genome. Discernible colony outgrowths were observed 4 weeks after infection, and clones were derived from emerging foci. Clonal expansion of individual single variant cells were subcultured, and six individual cell lines were retained. Among the six cell lines isolated, only one (Clo03) was selected for additional genetic and physiological characterization, on the basis of the clear polygonal epithelial-like morphology that these cells displayed in culture (see below). We first determined whether the endometrial transformed cells expressed the SV40 early genes. All nuclei of Clo03 cells were shown to contain T-antigen as revealed by indirect immunofluorescence staining (Figure 1A). Also, immunoblot analysis showed the presence of large T-antigen whereas a minor band corresponding to small T-antigen was also present (Figure 1B). We next analyzed the general pattern of SV40-specific DNA sequences in the transformed cells. High-molecular-weight cell DNA was extracted from Clo03 at passage 78 and analyzed by digestion with restriction endonucleases possessing different specificities for SV40 DNA. The results of these analyses are shown in Figure 2, and an unexpected observation was made. After digestion of genomic DNA with *Bam*HI restriction enzyme, which cleaves SV40 DNA at one point of the viral genome, a major band with a molecular weight of 5.2 kb was observed. Digestion with *Eco*RI, with one single restriction site in SV40, also exhibited a single band migrating at 5.2 kb. To determine whether this 5.2-kb band was generated from viral DNA molecules that were not integrated,

Figure 2. Analysis of SV40-specific DNA sequences in the genome of immortalized Clo03 endometrial cells. A: Cellular DNA (10 μ g) was digested with various restriction endonucleases, and the products of digestion were resolved on 0.8% agarose gels, transferred to nylon membrane, and hybridized with SV40 probe. B: Free viral DNA was isolated from nuclei of transformed cells and compared with purified SV40 DNA (6 pg) extracted from virions. FI, FII, and FIII indicate covalently closed circular superhelical SV40 DNA (form I), nicked circular relaxed DNA (form II), and linear DNA (form III), respectively. Asterisks indicate the position of closed circular superhelical SV40 DNA form.

the genomic DNA was digested with *Xba*I restriction enzyme, which lacks specificity for the SV40 genome but cleaves human cellular DNA. As illustrated in Figure 2A, a major hybridization signal (indicated by a star) with a faster electrophoretic mobility than would be expected for linearized SV40 was observed, suggesting a supercoiled close circular DNA. Additional evidence that the viral SV40 DNA existed in a free state was obtained by double digestion of the DNA with three combinations of two restriction enzymes, each of them cutting once in the viral genome: *Hpa*II plus *Bgl*II digestion resulted in 4.9- and 0.3-kb fragments; *Bam*HI plus *Bgl*II in 2.7- and 2.5-kb fragments; and *Bgl*II plus *Eco*RI in 3.4- and 1.7-kb fragments, as would be expected for restriction of full-length viral DNA. Finally, the presence of episomal SV40 DNA was confirmed by releasing these structures from isolated nuclei after mechanical homogenization in an isotonic buffer.¹⁶ Southern blot analyses (Figure 2B) showed indeed that the major band detected co-migrated with authentic supercoiled SV40 (form I) whereas a fainter band co-migrated as a nick circular DNA (form II). This latter form, which was not detected in total DNA (Figure 2A), could have been generated by relaxation of the supercoiled form after the extensive shearing forces necessary to make these molecules diffuse outside the nucleus. These results clearly indicated that SV40 DNA was present in a free state, implicating an autonomous mode of replication. Indeed, sequence analyses of the DNA fragment corresponding to the origin of replication present in the transformed cells showed identity with that

Figure 3. Morphological and immunocytochemical comparisons between immortalized endometriotic cells and primary endometriotic epithelial cells. **A:** Phase contrast microscopy showing polygonal cells with an epithelial-like morphology. Note that immortalized cells were less adherent to the bottom of the culture dish as compared with cells in primary cultures. Magnification, $\times 86$. **B to D:** Positive immunostaining for both immortalized and primary endometriotic cells with anti-cytokeratin antibodies (**B**), the BMA 180 antibody (**C**), and anti-vimentin antibody (**D**). Magnification, $\times 863$.

of wild-type SV40 (nucleotides 5191 to 5243/1 to 31).²⁴ The same results were observed whether DNA was extracted from cell cultures at early (13th) or late (78th) passages.

Immunofluorescence studies showed that, like primary endometriotic epithelial cells, immortalized cells positively reacted with anti-cytokeratin antibodies and with the BMA 180 antibody specific to a 200-kd epithelial cell glycoprotein. In immortalized cells, however, cytokeratins displayed a different pattern of structure, as they appeared more fragmented (less bundle shaped) as compared with untransformed cells that showed a filamentous network. In addition, both immortalized and primary endometriotic cells also showed a positive immunoreactivity with anti-vimentin antibody (Figure 3).

Karyotype

Chromosome analysis of 113 metaphases at passage 13 revealed that 96% of immortalized endometriotic cells had a polyploid karyotype. The distribution of chromosome number illustrated in Figure 4A shows a peak in the hypotetraploid region with 78% of metaphases containing 78 to 88 chromosomes. From the 113 metaphases

Figure 4. Chromosome number distribution of immortalized endometriotic cells from passages 13 and 78.

counted, 30 were photographed and analyzed. Figure 5A shows a GTG-banded polyploid karyotype of an immortalized cell from passage 13. The average percentage of mitoses with 1, 2, 3, or 4 copies of each autosome and X chromosome is shown in Figure 6. As can be seen, the mitoses analyzed have mainly three or four copies for most chromosomes. However, one or two copies of the autosomes 10, 11, 15, and 17 are found in more than 65% of the mitoses. Cytogenetic analysis also revealed chromosomal rearrangements, as shown in Table 2, which often involved autosomes 11 and 19.

Cytogenetic studies, including chromosome counting, karyotyping, and fluorescence *in situ* hybridization at passage 78 as compared with passage 13 showed hypertriploidy and most of the same chromosome rearrangements. For more than half of the metaphases analyzed, the number of chromosomes varied between 68 and 88 (Figure 4B). Also, chromosome rearrangements involving chromosomes 11 and 19 and low copy numbers of chromosomes 10, 15, and 17 were still found (data not shown). However, deletion of chromosome 6q with the breakpoint being at band 6q21 was observed in two of six metaphases karyotyped (Figure 5B).

Doubling Time

Immortalized endometriotic cells maintained in DMEM-F12 medium supplemented with 10% FBS-DC follow an exponential pattern of growth (Figure 7) with a comparable doubling time of 29 to 32 hours at early (15th) and late (78th) passages, estimated from direct viable cell count.

Expression of P and E₂ Receptors

According to the equation described in Materials and Methods, the number of E₂ and P receptors/cell, expressed as mean of triplicate determinations $\pm$ SE (SD), was 2656 ± 92 and 1799 ± 326 , respectively, at passage 15 and 3034 ± 286 and 1873 ± 106 , respectively, at passage 78.

Figure 5. A: GTG-banded karyotype of Clo03 cells from passage 13. Five marker chromosomes are identified as M1 to M5. B: At passage 78, one of the chromosomes 6 of two different metaphases shows a deletion at 6q21 (B).

Figure 6. Distribution of mitoses with 1, 2, 3, or 4 copies of each chromosome. Thirty GTG-banded mitoses were analyzed (passage 13).

Table 2. Structural Chromosome Rearrangements, Possible Chromosome Regions Involved, and Number of Mitoses in which the Abnormal Chromosomes Were Observed of 30 G-Banded Mitoses Analyzed (Passage 13)

Structural chromosome rearrangements	Chromosome regions	Number of mitoses
Dicentric*	dic (11; ?)	12
	dic (17; ?) [†]	8
	dic (10; ?) [†]	7
	dic (8; ?) [†]	5
	dic (5; ?) [†]	5
	dic (3; ?)	4
	dic (19; ?) [†]	4
	dic (12; ?) [†]	3
	dic (6; ?) [†]	3
	dic (4; ?) [†]	3
Deletion	del (11) (:q10→qter)	7
	del (11) (p?)	5
	del (5) (:q10→qter)	5
	del (21) (q?)	4
	del (7) (:q10→qter)	3
	del (6) (q?)	2
Translocation	t (2; 11) (q10; q10)	3
	t (X; 11) (q10; q10)	2
	t (7; 11) (q10; q10)	1
	t (3; 11) (p10; q10)	1
Isochromosome	i (19q)	5
	i (Xq)	3
	i (11q)	2
	i (7q)	1
Additional material of unknown origin	add (11p)	12
	add (19) (q13)	10
Ring		8

*Dicentric results principally from telomeric association of two chromosomes and, in some cases, contains additional material of unknown origin.

[†]Uncertain identification from the banding analysis. No *in situ* hybridization was carried out to confirm the chromosomal origin.

Figure 7. Growth curve of immortalized endometrial cells. Cells were seeded in six-well multiplates (15×10^3 cells/cm²) and cultured in DMEM-F12 medium supplemented with 10% FBS, 10 μ g/ml insulin, and 5 μ g/ml transferrin. Cells were dissociated with trypsin/EDTA and counted in triplicate, first after the 2-hour adhesion ($t = 0$) and then at different time intervals for a total of 168 hours.

Stimulation of MCP-1 Secretion by IL-1 β

We have recently shown that endometrial cells secrete high levels of MCP-1 in response to pro-inflammatory cytokines, such as IL-1 β ,¹¹ which are found in elevated levels in the peritoneal fluid of patients having endometriosis.^{3,5} This factor may play a significant role in monocyte recruitment into the peritoneal cavity and their activation. The objective of these experiments was therefore to assess the ability of an endometrial cell line to respond to the pro-inflammatory cytokine IL-1 β by secreting MCP-1. As shown in Figure 8, IL-1 β induced MCP-1 secretion in a time- and dose-dependent manner. MCP-1 secretion was detectable as early as 2 hours after incubation with 1 ng/ml IL-1 β and reached its highest level after 18 hours (Figure 8A). Culture stimulation with different concentrations of IL-1 β (0.01 to 10 ng/ml) during 6 hours resulted in a virtually linear elevation of MCP-1 concentrations measured in the culture supernatant (Figure 8B). The IL-1 β -induced MCP-1 secretion was significantly inhibited in a dose-dependent manner by dexamethasone (10^{-9} to 10^{-6} mol/L), a steroid anti-inflammatory agent that has been reported to inhibit MCP-1 secretion in a variety of cells (Figure 8C).

Human MCP-1 behaves on SDS-PAGE as multiple species with apparent molecular weights ranging from 8 to 18 kD, and it has been reported that differences in the processing of O-linked carbohydrates account for the heterogeneity of MCP-1 produced by different cell types.²⁵ Therefore, we examined the different MCP-1 species expressed by immortalized endometrial cells. After labeling the cells with [³⁵S]cysteine for 24 hours, proteins from the cell-free culture medium were immunoprecipitated with an anti-MCP-1-specific rabbit polyclonal antibody, and the isolated material was analyzed by SDS-PAGE followed by autoradiography. A representative analysis is shown in Figure 9, indicating that even in

Figure 8. Effects of IL-1 β and dexamethasone on MCP-1 secretion by immortalized endometriotic cells. **A:** Confluent cultures were incubated with 1 ng/ml IL-1 β for different time periods. **B:** Confluent cultures were incubated for 6 hours with varying concentrations of IL-1 β (0.01 to 10 ng/ml). **C:** Confluent cultures were incubated with different concentrations of dexamethasone (10⁻⁹ to 10⁻⁶ mol/L) for 18 hours before incubation with 1 ng/ml IL-1 β for an additional 6 hours. The levels of MCP-1 (pg/ml) secreted in the culture medium were measured by ELISA. Results are expressed as mean $\pm$ SEM of triplicate determinations.

the absence of any stimulation, endometriotic cells release detectable amounts of MCP-1, which appeared as three distinct bands with apparent molecular weights of 9, 13, and 15 kd. The intensity of these bands increased after stimulation of endometriotic cells with 0.01 to 1 ng/ml IL-1 β and decreased at higher doses (10 and 100 ng/ml). The observed bands were identified as distinct species of MCP-1 as their immunoprecipitation was ef-

Figure 9. SDS-PAGE analysis of MCP-1 secretion by immortalized endometriotic cells after stimulation for 24 hours with varying concentrations of IL-1 β . Lane 1, medium alone; lanes 2 to 6, 0.01 to 100 ng/ml IL-1 β ; lane 7, 1 ng/ml IL-1 β and 50 μ g/ml cycloheximide. In lane 8, cells were stimulated for 24 hours with 1 ng/ml IL-1 β , the supernatant was collected, and an excess of unlabeled MCP-1 (1 μ g/ml) was added before immunoprecipitation to inhibit the immunoprecipitation of [³⁵S]cysteine-labeled MCP-1 and to confirm, thereby, the specificity of MCP-1 antiserum for MCP-1.

fectively prevented in the presence of an excess of cold MCP-1 (1 μ g/ml), thereby confirming the specificity of the rabbit anti-MCP-1 antibody for MCP-1. It has previously been shown that this antibody does not cross-react with several cytokines that are closely related to MCP-1, including IL-8 and macrophage inflammatory proteins-1 α and -1 β .²⁶ Co-incubation of cells with IL-1 β (1 ng/ml) and cycloheximide (50 μ g/ml), an inhibitor of protein synthesis, resulted in an effective inhibition of MCP-1 expression (Figure 9).

Regulation of MCP-1 Secretion by Ovarian Steroids

It is known that endometriosis is dependent on ovarian steroids for its maintenance and development,²⁷ and numerous observations indicate that endometriotic tissue expresses ovarian steroid receptors, although at a lower level than the corresponding uterine endometrium.^{28,29} Based on this and on our results, described earlier, showing that immortalized cells express both E₂ and P receptors, we examined the responsiveness of an endometriotic cell line to ovarian hormones and the ability of these latter to modulate MCP-1 secretion by immortalized cells. Ovarian steroid doses (10⁻⁸ mol/L for E₂ and 10⁻⁶ mol/L for P) used in this study were selected on the basis of previous reports showing that ovarian steroid concentrations in the peritoneal fluid after ovulation are more elevated than those normally found in the peripheral blood.^{30,31} Treatment of immortalized cells with E₂ and P resulted in a significant increase of spontaneous secretion of MCP-1 in culture (Figure 10A). This effect was more obvious when MCP-1 secretion was amplified by IL-1 β stimulation (Figure 10B). Co-incubation of cells with E₂ and P resulted in an additional increase of MCP-1 secretion compared with each hormone alone. This was also observed with primary endometriotic epithelial cell cultures (Figure 10C), indicating that the endometriotic cell line retains differentiated functions and reacts to IL-1 β and to ovarian hormones by secreting MCP-1 in a manner comparable to parental cells. It is worthy of note that the effect of ovarian hormones on MCP-1 secretion by primary cultures was investigated only with cytokine-

Figure 10. Effects of E₂ (10⁻⁸ mol/L) and P (10⁻⁶ mmol/L) on spontaneous (A) and IL-1β-induced (B) secretion of MCP-1 by immortalized endometriotic cells. C: Effects of E₂ (10⁻⁸ mmol/L) and P (10⁻⁶ mmol/L) on IL-1β-induced secretion of MCP-1 by primary cultures of epithelial endometriotic cells. Hormones were added to the culture medium from the culture initiation. The culture medium was changed every 2 days until confluence was reached. FBS was then discarded, and incubation with hormones was pursued for 48 hours (spontaneous secretion) or for 42 hours followed by 6 hours of stimulation with 1 ng/ml IL-1β (IL-1β-induced secretion). MCP-1 concentrations were evaluated by ELISA. The results were expressed as the percentage above or below control (without treatment with hormones). For immortalized cells, values are means ± SEM of triplicate determinations. For primary endometriotic cells, values are means ± SEM of duplicate determinations in three different patients. Significantly different from control: **P* < 0.05 and ***P* < 0.01 by the Tukey's multiple comparison test.

stimulated cells because of the low number of epithelial cells that can be isolated from endometriotic biopsies.

The cells' responsiveness to IL-1β and to steroid hormones in terms of MCP-1 secretion was also assessed at a late passage (78th), and the results showed a pattern of action comparable to that described earlier for cells from an early passage (15th) (data not shown).

Discussion

Endometriosis is one of the major and the most frequent gynecological disorders. It represents the unique benign condition where autologous cells, taking origin from the endometrium, according to the most predominant hypothesis,³² migrate and develop at abnormal locations. It is believed that ectopic endometrial tissue might contribute to the biological changes observed both locally and systemically in patients having endometriosis^{9-13,33} and perhaps to endometriosis-associated infertility.³⁴ The few

available studies regarding ectopic endometrial cell functions are based on the use of whole tissue or, very often, endometriotic stromal cells because of their predominance in endometriotic tissue and their growth characteristics *in vitro* (short doubling time and relatively easy to culture, to maintain, and to expand for several passages). Endometriotic tissue that is adequate for cell culture is, however, not always available, and it is often very difficult to recover the amount of epithelial cells required for extended studies on these cells.

We report here a cytogenetic and physiological study of an endometriotic cell line that was immortalized by viral infection of primary epithelial endometriotic cell cultures with SV40. As previous attempts to immortalize endometriotic cell with different vectors have failed, we used in a last attempt viral infection that has been successfully used to establish human cell lines.^{35,36} Six lines derived from six individual foci were isolated. Taking into account that the establishment of permanently transformed cell lines after infection with SV40 is a very rare event and requires nonlethal insertion of viral genetic material into host chromosomal DNA and expression of a functional SV40 early gene,³⁶⁻³⁸ it is puzzling that starting with only 10 × 10⁴ endometrial cells in our transformation assays as many as six clones were obtained. Three hypotheses can be proposed to explain such a high rate of success. First, endometriotic cells may be particularly sensitive to SV40 transformation. However, attempts to transform several other primary endometriotic cell cultures from different patients have failed. Alternatively, it is possible that the particular explant of patient 1 was in a physiological state in the proliferative phase of the menstrual cycle that might have rendered cells susceptible to viral infection (Table 1). Finally, the six clones may in fact not be independent and would rather emerge from a single transformed cell. Work is in progress to determine whether the other selected clones, namely, 06, 11, 12, 14, 15, and 16 are sister lines or not of Clo03.

Our Southern blot analyses clearly showed that SV40 DNA is episomal in Clo03 cells. The possibility that the linear 5.2-kb SV40 fragment results from an integrated double dimers of SV40 is ruled out by the digestion with the non-cutter restriction enzyme *Xba*I, which does not result in high-molecular-weight DNA as is the case in SV40-induced mesotheliomas in hamsters.³⁹ In addition, no convincing hybridization signals for integrated SV40 molecules could be shown using the different cutter and non-cutter SV40-specific restriction enzymes. However, it is widely believed that SV40 DNA must be integrated into the host cell DNA to transform human cell lines,³⁶⁻³⁸ and it is inferred that episomal SV40, as seen in rodent cells and human keratinocytes,^{40,41} might be the consequence of extensive post-integration lability of the viral genome leading to the excision of the integrated fragment by recombination mechanisms^{37,42} and might be considered as an epiphenomenon.³⁷ Recently, SV40-like sequences have been found associated with several types of human tumors: mesotheliomas, osteosarcomas, ependymomas, and brain tumors (reviewed in Ref. 43). The possibility that SV40 played a causative role in these human malignancies has been considered,⁴³⁻⁴⁵ and the

presence of episomal SV40 DNA in some of these tumors has been reported.⁴⁶⁻⁴⁹ The persistence of free viral forms as observed in Clo03 endometriotic cells at early as well as at late passages is puzzling and may indicate that the free DNA has acquired an apparent stable state in these cell lines. It is therefore tempting to conclude that our *in vitro* experiment substantiates the possibility that episomal SV40 could indeed transform human cells and that this phenomenon might be cell type dependent. This of course does not rule out the possibility that during the very initial steps of transformation, the viral DNA has once been integrated and that the free viral DNA arises from excision of molecules from the host genome as is observed in non-permissive rodent cells.⁴²

At an early passage of culture, the cells exhibited an overall hypotetraploid karyotype with a frequent presence of only one or two copies for chromosomes 10, 11, 15, and 17 in the mitoses analyzed. At a late passage, the cells showed a hypertriploid karyotype, but cytogenetic analyses revealed comparable chromosome rearrangements, mainly involving the same chromosomes. T-antigen has been reported to induce successive rounds of DNA synthesis without intervening mitosis in transformed cells, leading to polyploidy,^{50,51} and to cause karyotypic destabilization and instability.^{35,52} However, to explain the low number of copies for chromosomes 10, 11, 15, and 17, the following hypotheses could be put forward: 1) these chromosomes were involved in the early stages of chromosome rearrangements; 2) they were preferentially lost because they contain genes deleterious for cell growth when present in too many copies; or 3) the endometriotic cells that became immortalized had only one copy of those chromosomes.

Immortalized cells showed a strong positive immunoreactivity for cytokeratins, which are known to be expressed only by epithelial cells, and reacted positively with an antibody that specifically recognizes a 200-kd glycoprotein expressed by epithelial endometrial/endometriotic cells.¹⁹ This suggests that the cell line is of epithelial-like nature. However, like primary endometriotic epithelial cells, immortalized cells were also immunoreactive with anti-vimentin. Available data regarding the specificity of vimentin as a marker for stromal cells in culture are contradictory.^{13,53-55} However, initiation of vimentin expression seems to be an adaptation to cell culture conditions rather than being indicative of mesenchymal cell origin.⁵⁶

The endometriotic cell line showed the ability to spontaneously secrete MCP-1, a potent monocyte chemoattractant and activating factor. That secretion was significantly enhanced by IL-1 β , a major pro-inflammatory cytokine found in elevated concentrations in the peritoneal cavity of patients with endometriosis,^{3,5} and was inhibited by dexamethasone, an anti-inflammatory steroid hormone that also inhibits MCP-1 secretion by primary endometriotic cell cultures (A. Akoum, unpublished results). These results are in line with our previous studies showing that after stimulation with pro-inflammatory cytokines *in vitro*, ectopic as well as eutopic primary endometrial cells of women with endometriosis secrete increased amounts of MCP-1.^{11,57} This factor may play an important

role in the activation of peripheral blood monocytes and peritoneal macrophages in endometriosis,^{7,58} and, according to our recent findings, the up-regulation of its expression arises even *in situ* in the intrauterine endometrium of endometriosis patients.⁵⁹ Three electrophoretically distinct species of MCP-1 with estimated molecular weights of 9, 13, and 15 kd were detected in the culture medium, which is also consistent with our previous data regarding MCP-1 expression by primary cultures of endometriotic and endometrial cells.^{11,57} On SDS-PAGE, MCP-1 behaves as multiple species with molecular weights ranging from 8 to 18 kd. The core protein of approximately 8 kd may be synthesized by all cells that produce MCP-1. However, this protein undergoes glycosylation, which, according to the cell type, results in the appearance of proteins having different molecular weights.²⁵

We have also shown that immortalized endometriotic cells express P and E₂ receptors. Furthermore, they were directly stimulated to secrete MCP-1 by ovarian steroids, a response that was enhanced in IL-1 β -treated cells. The cytokine-induced secretion of MCP-1 was significantly increased by E₂ (10⁻⁸ mol/L) and also by P (10⁻⁶ mol/L), although to a lesser extent, a response that, interestingly, was observed with primary endometriotic epithelial cells as well. Moreover, both in immortalized and primary endometriotic cells, MCP-1 secretion was further enhanced by E₂ and P together. These findings clearly show that ovarian hormones exert a synergistic action on MCP-1 secretion by endometriotic cells and suggest a cycle-dependent control of MCP-1 expression by endometriotic lesions. They also provide for the first time evidence that ovarian steroids may contribute to the liberation of a potent and specific mediator of monocyte chemoattraction and activation into the peritoneal cavity of patients with endometriosis. On the other hand, these findings indicate that the cell line is responsive to ovarian steroids and to pro-inflammatory stimuli, which, interestingly, was observed even after a long-term cell culture. They also indicate that, at least with regard to the IL-1 β -dependent and the ovarian-hormone-dependent MCP-1 secretion, the line reacts in a manner comparable to that of normal non-immortalized endometriotic cells and retains differentiated functions. Although interesting, this nevertheless was surprising in view of the polyploidy of the cell line and cannot be explained with certainty. One potential explanation might presumably lie in the fact that the MCP-1 gene is located on chromosome 17 (17q11.2-q21.1),⁶⁰ which, according to our cytogenetic analysis, did not show detectable structural rearrangements. On the other hand, numerous cell lines immortalized with SV40 T-antigen have been reported to retain differentiated functions as compared with the parental cells.⁶¹⁻⁶⁴

The cell line Clo03 has been maintained in culture for over 80 passages with no evidence of cell crisis. Cells were still proliferating without any noticeable change in the population doubling time or in cell morphology, suggesting that they are immortal, and cytogenetic analysis of cells from passage 78 showed deletions in the long arm of chromosome 6, which has recently been shown to harbor growth suppressor or senescence-related

genes^{65,66} and could therefore account for the immortalization of the endometriotic cell line.

In summary, the findings reported in the present study support the view that the immortalized Clo03 cell line may be an interesting tool for the study of the pathophysiology of endometriosis. In addition, finding episomal SV40 in an SV40-transformed human cell line, and finding the characteristic markers of SV40-mediated transformation in the same line with nuclear T-antigen expression and genetic alterations, including that of 6q, may be of great interest for the understanding of SV40 transformation of human cells.

Acknowledgments

We express our appreciation to the referees for their constructive comments during the review process. We thank Dr. Peter Gronski, Centeon Pharma GmbH, Marburg, Germany, for providing us with the mouse monoclonal BMA 180 antibody; Dr. Ed Harlow for PABs 414 and 419; Drs. Jacques Bergeron and Marc Villeneuve for patient evaluation and for providing endometriotic biopsies; Marc Bronsard, Isabelle Paradis, and Sandra Tremblay for technical assistance; and Dr. Mahéra Al-Akoum for the critical review of the manuscript.

References

1. Haney AF, Muscato JJ, Weinberg JB: Peritoneal fluid cell populations in infertility patients. *Fertil Steril* 1981, 35:696–698
2. Halme J, Becker S, Hammond MG, Raj MHG, Raj S: Increased activation of pelvic macrophages in infertile women with mild endometriosis. *Am J Obstet Gynecol* 1983, 145:333–337
3. Fakh H, Baggett B, Holtz G, Tsang KY, Lee JC, Williamson HO: Interleukin-1: a possible role in the infertility associated with endometriosis. *Fertil Steril* 1987, 47:213–217
4. Eisermann J, Gast MJ, Pineda J, Odem RR, Collins JL: Tumor necrosis factor in peritoneal fluid of women undergoing laparoscopic surgery. *Fertil Steril* 1988, 50:573–579
5. Taketani Y, Kuo TM, Mizuno M: Comparison of cytokine levels and embryo toxicity in peritoneal fluid in infertile women with untreated and treated endometriosis. *Am J Obstet Gynecol* 1992, 167:765–770
6. Khorram O, Taylor RN, Ryan IP, Schall TJ, Landers DV: Peritoneal fluid concentrations of the cytokine RANTES correlate with the severity of endometriosis. *Am J Obstet Gynecol* 1993, 169:1545–1549
7. Akoum A, Lemay A, McColl S, Turcot-Lemay L, Maheux R: Elevated concentration and biological activity of monocyte chemotactic protein-1 in the peritoneal fluid of patients with endometriosis. *Fertil Steril* 1996, 66:17–23
8. Vernon MW, Beard JS, Graves K, Wilson EA: Classification of endometriotic implants by morphologic appearance and capacity to synthesize prostaglandins. *Fertil Steril* 1986, 46:801–806
9. Isaacson KB, Coutifaris C, Garcia CR, Lyttle CR: Production and secretion of complement component 3 by endometriotic tissue. *J Clin Endocrinol Metab* 1989, 69:1003–1009
10. Shifren JL, Tseng JF, Zaloudek CJ, Ryan IP, Gloria Meng Y, Ferrara N, Jaffe RB, Taylor RN: Ovarian steroid regulation of vascular endothelium growth factor in the human endometrium: implications for angiogenesis during the menstrual cycle and in the pathogenesis of endometriosis. *J Clin Endocrinol Metab* 1996, 81:3112–3118
11. Akoum A, Lemay A, Brunet C, Hébert J: Cytokine-induced secretion of monocyte chemotactic protein-1 by human endometriotic cells in culture. *Am J Obstet Gynecol* 1995, 172:594–600
12. Akoum A, Lemay A, Rheault N, Paradis I, Maheux R: Secretion of interleukin-6 by endometriotic cells and regulation by inflammatory cytokines and sex steroids. *Hum Reprod* 1996, 10:101–107
13. Rier SE, Zarmakoups PN, Hu X, Becker JL: Dysregulation of IL-6 responses in ectopic endometrial stromal cells: correlation with decreased soluble receptor levels in peritoneal fluid of women with endometriosis. *J Clin Endocrinol Metab* 1995, 80:1431–1437
14. Khandjian EW, Gauchat J-F: One single burst of SV40 gene expression induces cell proliferation in transforming infection of mouse cells. *Oncogene* 1988, 3:195–200
15. Gross-Bellard M, Oudet P, Chambon P: Isolation of high-molecular-weight DNA from mammalian cells. *Eur J Biochem* 1973, 36:32–38
16. Türlér H, Beard P: Simian virus 40 and polyoma virus: growth, titration, transformation and purification of viral components. *Virology, A Practical Approach*. Edited by Mahy BWH. Oxford, IRL Press, pp 169–192
17. Khandjian EW: Optimized hybridization of DNA blotted and fixed to nitrocellulose and nylon membranes. *BioTechnology* 1987, 5:165–167
18. Harlow E, Crawford LV, Pim DC, Williamson NM: Monoclonal antibodies specific for simian virus 40 tumor antigens. *J Virol* 1981, 39:861–869
19. Kruitwagen RFFM, Poels LG, Willemsen WNP, Jap PHK, de Ronde IJY, Hanselaar TGJM, Rolland R: Immunocytochemical marker profile of endometriotic epithelial, endometrial epithelial, and mesothelial cells: a comparative study. *Eur J Obstet Gynecol Reprod Biol* 1991, 41:215–223
20. Khandjian EW: Heat treatment induces dephosphorylation of the retinoblastoma gene product and dissociation of T-antigen/pRb protein complex during transforming infection with SV40. *Oncogene* 1995, 10:359–367
21. Ikeuchi T: Inhibitory effect of ethidium bromide on mitotic chromosome condensation and its application to high-resolution chromosome banding. *Cytogenet Cell Genet* 1984, 38:56–61
22. Drouin R, Lemieux N, Richer CL: High-resolution R-banding at the 1250-band level 1: technical considerations on cell synchronization and R-banding (RHG and RBG). *Cytobios* 1988, 56:107–125
23. Asselin J, Kelly PA, Caron MG, Labrie F: Control of hormone receptor levels and growth of 7,12-dimethylbenz(a)anthracene-induced mammary tumors by estrogens, progesterone and prolactin. *Endocrinology* 1977, 101:666–671
24. Tooze J: The molecular biology of tumor viruses. II. DNA tumor viruses. The SV40 Nucleotide Sequence, Appendix A. Cold Spring Harbor, NY, Cold Spring Harbor Monograph, 1980, pp 799–829
25. Jiang Y, Valente AJ, Williamson MJ, Zhang L, Graves DT: Post-transcriptional modification of a monocyte specific chemoattractant synthesized by glioma, osteosarcoma and vascular smooth muscle cells. *J Biol Chem* 1990, 265:18318–18321
26. Hachicha M, Rathanaswami P, Schall TJ, McColl SR: Production of monocyte chemotactic protein-1 in human type B synovialocytes. *Arthritis Rheum* 1993, 36:26–34
27. Di zerga GS, Barger DL, Hodgen GD: Endometriosis: role of ovarian steroids in initiation, maintenance and suppression. *Fertil Steril*, 1980, 33:649–653
28. Bergqvist A, Ljungberg O, Skoog L: Immunohistochemical analysis of oestrogen and progesterone receptors in endometriotic and endometrium. *Hum Reprod* 1993, 8:1915–1922
29. Prentice A, Randall BJ, Weddell A, McGill A, Henry L, Horne CHW, Thomas EJ: Ovarian steroid receptor expression in endometriosis and in two potential parent epithelia: endometrium and peritoneal mesothelium. *Hum Reprod* 1992, 9:1318–1325
30. De Leon FD, Vijayakumar R, Brown M, Rao CHV, Yussman MA, Schultz G: Peritoneal fluid volume, estrogen, progesterone, prostaglandin, and epidermal growth factor concentrations in patients with and without endometriosis. *Obstet Gynecol* 1986, 68:189–194
31. McLaren J, Prentice A, Charnock-Jones DS, Millican SA, Muller KH, Sharkey AM, Smith SK: Vascular endothelial growth factor is produced by peritoneal fluid macrophages in endometriosis and is regulated by ovarian steroids. *J Clin Invest* 1996, 98:482–489
32. Sampson JA: Peritoneal endometriosis due to the menstrual dissemination of endometrial tissue into the peritoneal cavity. *Am J Obstet Gynecol*, 1927, 14:422–469
33. Halme J: Role of peritoneal inflammation in endometriosis-associated infertility. *Ann NY Acad Sci* 1991, 6:266–274
34. Marcoux S, Maheux R, Bérubé S, The Canadian Collaborative Group on Endometriosis: Laparoscopic surgery in infertile women with minimal or mild endometriosis. *N Engl J Med* 1997, 337:217–222

35. Chang SE: In vitro transformation of human epithelial cells. *Biochim Biophys Acta* 1986, 823:161–194
36. Bryan TM, Reddel RR: SV40-induced immortalization of human cells. *Crit Rev Oncogenesis* 1994, 5:331–357
37. Monier R: Transformation by SV40 and polyoma. The Papovaviridae, vol 1: The Polyomaviruses. New York, Plenum Press, 1986, pp 247–294
38. Martin RG: The transformation of cell growth and transmutation of DNA synthesis by simian virus 40. *Adv Cancer Res* 1981, 34:1–68
39. Ciccalà C, Pompetti F, Carbone M: SV40 induces mesotheliomas in hamsters. *Am J Pathol* 1993, 142:1524–1533
40. Hiscott JB, Murphy D, Defendi V: Instability of integrated viral DNA in mouse cells transformed by simian virus 40. *Proc Natl Acad Sci USA* 1981, 78:1736–1740
41. Naimski P, Steinberg ML: Analysis of stable and unstable viral forms in SV40-infected human keratinocytes. *J Cell Biochem* 1985, 29:95–103
42. Bullock P, Forrester W, Botchan M: DNA sequence studies of simian virus 40 chromosomal excision and integration in rat cells. *J Mol Biol* 1984, 174:55–84
43. Carbone M, Rizzo P, Pass HI: Simian virus 40, poliovaccines and human tumors: a review of recent developments. *Oncogene* 1997, 15:1877–1888
44. Wiman KG, Klein G: An old acquaintance resurfaces in human mesothelioma. *Nature Med* 1997, 3:839–840
45. Stenton SC: Simian virus 40 and human malignancy. *Br Med J* 1998, 316:877
46. Butel JS, Jafar S, Stewart AR, Lednický JA: Detection of authentic SV40 DNA sequences in human brain and bone tumors. *Dev Biol Stand* 1998, 94:23–32
47. Lednický JA, Garcea RL, Bergsagel DJ, Butel JS: Natural simian virus 40 strains are present in human choroid plexus and ependymoma tumors. *Virology* 1995, 212:710–717
48. Krieg P, Scherer G: Cloning of SV40 genomes from human brain tumors. *Virology* 1984, 138:336–340
49. Krieg P, Amtmann E, Jonas D, Fisher H, Zang K, Sauer G: Episomal simian virus 40 genomes in human brain tumors. *Proc Natl Acad Sci USA* 1981, 78:6446–6450
50. Friedrich TD, Laffin J, Lehman, JM: Induction of tetraploid DNA content by simian virus 40 is dependent on T-antigen function in the G2 phase of the cell cycle. *J Virol* 1994, 68:4028–4030
51. Rinehart CA, Mayben JP, Butler, TD, Haskill JS, Kaufman DG: Alterations of DNA content in human endometrial stromal cells transfected with a temperature-sensitive SV40: tetraploidization and physiological consequences. *Carcinogenesis* 1992, 13:63–68
52. Ray FA, Peabody DS, Cooper JL, Cram LS, Kraemer PM: SV40 T antigen alone drives karyotype instability that precedes neoplastic transformation of human diploid fibroblasts. *J Cell Biochem* 1990, 42:13–31
53. Sharpe KL, Zimmer RL, Griffin WT, Penney LL: Polypeptides synthesized and released by human endometriosis differ from those of the uterine endometrium in cell and tissue explant culture. *Fertil Steril* 1993, 60:839–851
54. Matthews CJ, Redfern CPF, Hirst BH, Thomas EJ: Characterization of human purified epithelial and stromal cells from endometrium and endometriosis in tissue culture. *Fertil Steril* 1992, 57:990–997
55. Ryan IP, Schriock ED, Taylor RN: Isolation, characterization, and comparison of human endometrial and endometriosis cells *in vitro*. *J Clin Endocrinol Metab* 1994, 78:642–649
56. Franke WW, Schmid E, Winter S, Osborn M, Weber K: Widespread occurrence of intermediate sized filaments of the vimentin type in cultured cells from diverse vertebrates. *Exp Cell Res* 1979, 132:25–46
57. Akoum A, Lemay A, Brunet C, Hébert J: Secretion of monocyte chemotactic protein-1 by cytokine-stimulated endometrial cells of women with endometriosis. *Fertil Steril* 1995, 63:322–328
58. Akoum A, Lemay A, McColl SR, Paradis I, Maheux R: Increased monocyte chemotactic protein-1 level and activity in the peripheral blood of women with endometriosis. *Am J Obstet Gynecol* 1996, 175:1620–1625
59. Jolicoeur C, Boutouil M, Drouin R, Paradis I, Lemay A, Akoum A: Increased expression of monocyte chemotactic protein-1 in the endometrium of women with endometriosis. *Am J Pathol* 1998, 152:125–133
60. Mehrabian MRSI, Sparkes TM, Fogelman AM, Lusk AJ: Localization of monocyte chemotactic protein-1 gene (SCYA2) to human chromosome 17q11.2-q21.1. *Genomics* 1991, 9:200–203
61. Brosens JJ, Takeda S, Acevedo CH, Lewis MP, Kirby PL, Symes EK, Krausz T, Purohit A, Gellersen B, White JO: Human endometrial fibroblasts immortalized by simian virus 40 large T antigen differentiate in response to a decidualization stimulus. *Endocrinology* 1996, 137:2225–2231
62. Wiehle RD, Helftenbein G, Land H, Neuman K, Beato M: Establishment of rat endometrial cell lined by retroviral mediated transfer of immortalizing and transforming oncogenes. *Oncogene* 1990, 5:787–794
63. Srivastava RK, Gu Y, Zilberstein M, Ou JS, Mayo KE, Chou JY, Gibori G: Development and characterization of a simian virus 40-transformed, temperature-sensitive rat antimesometrial decidual cell line. *Endocrinology* 1995, 136:1913–1919
64. Jacob JR, Estlack LE, Lanford RE: Immortalization of chimpanzee hepatocytes with an amphoteric retrovirus encoding simian virus T antigen. *Exp Cell Res* 1992, 200:205–210
65. Hubbard-Smith K, Patsalis P, Pardinis JR, Jha KK, Henderson AS, Ozer HL: Altered chromosome 6 in immortal human fibroblasts. *Mol Cell Biol* 1992, 12:2273–2281
66. Banga SS, Kim S, Hubbard K, Dasgupta T, Jha KK, Patsalis P, Hauptschein R, Gamberi B, Dalla-Favera R, Kraemer P, Ozer HL: SEN6, a locus for SV40-mediated immortalization of human cells, maps to 6q26–27. *Oncogene* 1997, 14:313–321